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The *Pseudomonas* phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants

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Abstract

The phytotoxic principle, coronatine, which is present in several pathovars of the plant pathogen, *Pseudomonas syringae* was shown to be highly active in completely different, jasmonate-selective bioassays. At nanomolar to micromolar concentrations, coronatine induced the accumulation of defense-related secondary metabolites in several plant cell cultures, induced transcript accumulation of the elicitor-responsive gene encoding the berberine bridge enzyme of *Eschscholtzia californica*, as well as the coiling response of *Bryonia dioica* tendrils. Biological activity critically depended upon the structure of coronatine, and slight modifications, such as methylation of the carboxyl moiety or reduction of the carbonyl group, rendered the molecules almost inactive. Coronafacic acid, obtained by hydrolysis of coronatine, was also nearly inactive. Coronatine did not elicit the accumulation of endogenous jasmonic acid in the systems analyzed. While coronafacic acid is similar in structure to jasmonic acid, we found coronatine to be a close structural analogue of the cyclic C18-precursor of jasmonic acid, 12-oxo-phytodienoic acid. The phytotoxic symptoms produced by coronatine can now be understood on the basis of the toxin's action as a mimic of the octadecanoid signalling molecules of higher plants.

Key words: Coronatine; Jasmonic acid; Methyljasmonate; 12-Oxo-phytodienoic acid; Coronatine (mode of action)

1. Introduction

Coronatine (**1**) is a bacterial blight toxin produced by several pathovars of *Pseudomonas syringae* von Hall which induces chlorosis, increased ethylene evolution and accelerated senescence associated with the disease [1,2] that affects several host plants, including agronomically important species such as soybean [3]. Despite earlier attempts [2,4] at elucidation, the mode of action of coronatine remains unknown. The toxin is an amide of coronafacic acid and 2-ethyl-1-aminocyclopropane-1-carboxylic acid (coronamic acid) [5], with both moieties being indispensable for the compound's phytotoxic activity [6].

Recently, it has been shown that jasmonic acid (JA, **10**) has weak, coronatine-like activity in the potato tuber tissue expansion assay, the most sensitive bioassay available to determine the presence of coronatine [3,7,8]. Structural similarity between coronafacic acid (**3**) and (3*R*,7*S*)-JA, the isomer thought to be biosynthesized by higher plants, has also been noted [8]. In the upper micromolar range, JA accelerates senescence and chlorophyll degradation and induces ethylene evolution (for review see [9]), symptoms resembling those of coronatine toxicity. At lower levels, however, JA is a potent signal transducer in the plant's defense against herbivore [10] or pathogen [11] attack and is involved in mechanotransduction [12]. The latter two processes provide exceptionally sensitive and specific biological assays for octadecanoid-derived plant signalling molecules such as JA. We report here that coronatine is highly active in these assays, but does not elicit the accumulation of endogenous JA in treated tissues. Inspection of the structure of coronatine has revealed that the compound is a close structural analogue of the jasmonate biosynthetic precursor, 12-oxo-phytodienoic acid (**6**). The data reported herein establish the mode of action of coronatine as a mimic of octadecanoid-derived signalling in higher plants.

2. Materials and methods

Plant materials were grown as described [11–14]. Treatment of tissue cultures with methyljasmonate (MeJA, **11**) or coronatine as well as determinations of secondary metabolite production and of JA levels were as reported earlier [11,13], the tendril bioassay was carried out exactly as described in [14]. Berberine bridge enzyme transcripts were analyzed as in [15]. Coronatine was produced and purified according to [16]. Hydrolysis of the amide and isolation of coronafacic acid [17] was followed by purification by HPLC [16]. The product was 90% cis and 10% trans-isomer as determined by ^{1}H NMR using the assignments reported in [5]. The purity of coronatine and coronafacic acid were finally checked by GC-MS and capillary electrophoresis. For indicated experiments, HPLC-purified coronatine was converted to the methyl ester (**2**), purified by HPLC, then base-hydrolyzed to yield coronatine, which was purified once-more by HPLC. Carbonyl reductions, methylations and the sources of all jasmonates are reported in [14]. All structures and purities were verified by capillary GC-MS based on both EI (70 eV) and CI (methanol) ionization modes and full-scan spectral information.

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3. Results

The dose–response relationships for (1), its derivatives and different endogenous octadecanoids compared with that for (11) in the tendril curvature assay, so far the most specific and rapid jasmonate bioassay known [14], are shown in Fig. 1. The structures and numbering for all compounds used in this study are given in Fig. 2. In this bioassay, test compounds are usually applied as methyl esters which load better into the tissue and thus give a faster as well as stronger response [12,14]. (1) provides a notable exception in that its methyl ester (2) is almost inactive (see insert in Fig. 1) while (1) is the most active molecule so far found for this system. Since we used (1) isolated from a natural source [16], it was essential to establish the absence of any cross-contaminant. The chemical conversion of (1) to (2), followed by HPLC purification, then base hydrolysis of (2) back to (1) with subsequent purification by HPLC showed that the biological activity was tightly associated with (1) throughout the sequence of conversions and purifications (cf. insert in Fig. 1).

Addition of (1) to several plant cell cultures known to respond to both microbial elicitors and jasmonates with the accumulation of low molecular weight defense molecules [11,13,15] proved that (1) was also clearly active in these systems (Figs. 3 and 4). The accumulation of benzo[*c*]phenanthridine alkaloids in cell cultures of *Eschscholtzia californica* as a function of concentration of (1) or (11) and the time course of response to (1) are shown in Fig. 3A and B. While the threshold level of activity was about 50- to 100-fold lower for (1) as for (11), the maximum response to (11) was stronger. A different situation was found in cell cultures from other species (cf. Fig. 4). The accumulation of indole alkaloids by cells of *Rauvolfia serpentina* in the presence of as little as 1 μM (1) was stronger than that induced by 100 μM (11) (cf. Fig. 4A and B), and as little as 100 nM (1) elicited a maximum accumulation of isoflavones in cell cultures of *Glycine max* (cf. Fig. 4C). In all cases, a complex spectrum of defense-related secondary metabolites induced by (1) was similar to that induced by a yeast elicitor or (11) [11] (cf. Fig. 4A and B).

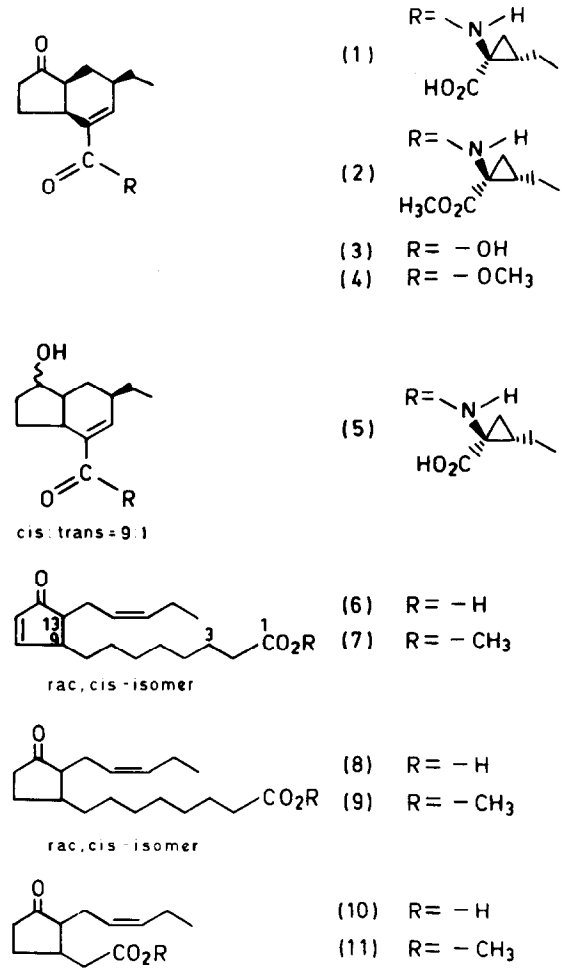

Fig. 2. Structure of compounds used in the present study.

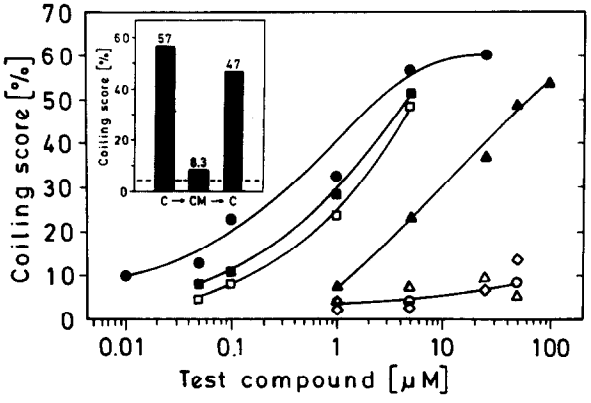

Fig. 1. Activity of coronatine, coronatine derivatives and jasmonates in inducing tendril coiling in *Bryonia dioica*. Symbols indicate the individual test compounds as follows (average data from *n* separate experiments): ●, **1** (coronatine, *n* = 5); ■, **7** (12-oxo-phytodienoic acid methyl ester, *n* = 5); □, **9** (12-oxo-dihydrophytodienoic acid methyl ester, *n* = 3); ▲, **11** (methyl jasmonate, *n* = 25); ○, **5** (*n* = 3); △, **3** (coronafacic acid, *n* = 4); ◇, **4** (coronafacic acid methyl ester, *n* = 4). The insert shows the sequentially determined biological activity of the original sample of coronatine (C), after its conversion to the methyl ester (CM) and after its hydrolysis back to coronatine (C); all compounds tested at 5 μM final concentration; data averaged from two independent experiments. Given for all data points is the coiling score after 8 h of incubation [14].

Berberine bridge enzyme transcript accumulated in a concentration-dependent manner in cell suspension cultures treated with (1) (Fig. 5A). This tightly regulated enzyme catalyzes a central step in the biosynthesis of the antimicrobial benzo[*c*]phenanthridine alkaloids in *Eschscholtzia*. The transcription of its gene has been shown to be strongly responsive to fungal elicitors, jasmonates and their octadecanoid precursors ([15] and references cited therein). Likewise, berberine bridge enzyme activity accumulated in (1)-induced cell suspension cultures (Fig. 5B), but to a maximal level that was 20-fold less than that typically induced by 100 μM (11) [18]. Even with these

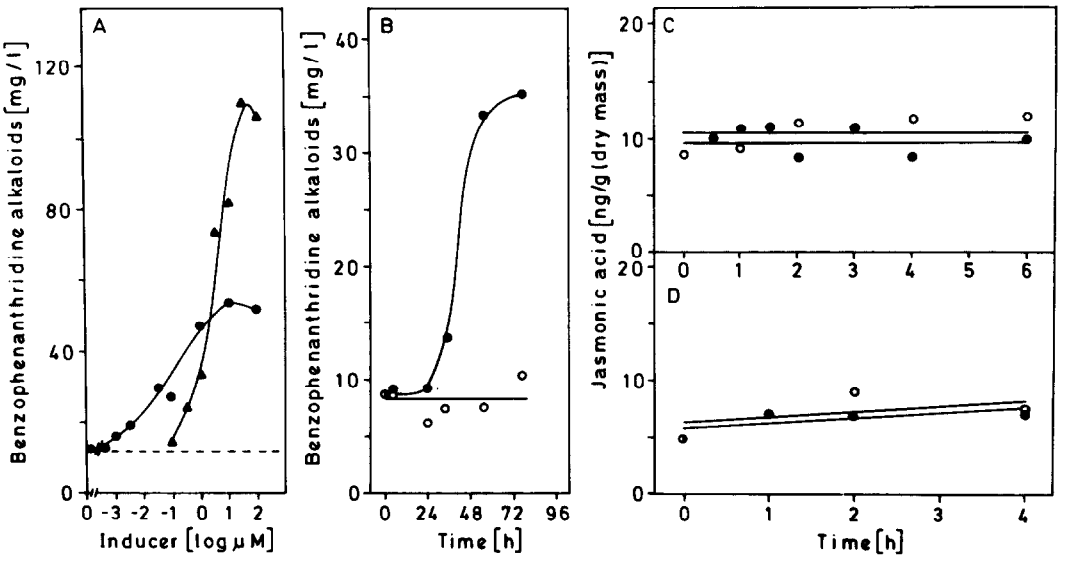

Fig. 3. Coronatine action on cell cultures of *Eschscholtzia californica* (A–C) and *Agrostis tenuis* (D). (A) Benzophenanthridine alkaloid accumulation as a function of concentration of coronatine (●) or methyljasmonate (▲); dashed line: alkaloid level in untreated control cells. (B) Time course of alkaloid accumulation in control cells (○) or cells treated with 5 μM coronatine (●). (C) time course of jasmonic acid levels of control cells (○) or cells treated with 5 μM (C) or 20 μM (D) coronatine (●). Alkaloid data expressed as amount per liter of cell culture harvested where one liter = 5.8 g of dry mass. All data averaged from two independent experiments. Experimental details as in [11,13].

reduced levels of berberine bridge enzyme induced by (1) as compared to induction by (11), total benzophenanthridine alkaloids produced in response to (1) accumulated to 40% of those values obtained by elicitation with (11).

Whereas microbial elicitors induce a rapid, massive and transient accumulation of JA (10) in all cell culture systems used here [13], coronatine (1) cannot be classified as an elicitor, because it stimulated JA accumulation neither in *E. californica* nor in *Agrostis tenuis* cell cultures (Fig. 3C,D). This latter species shows the most dramatic, several-hundred-fold rise in JA-levels following elicitation so far known ([13]; Fig. 1A). Likewise, tendril coiling induced by (1) proceeded without any alteration in the level of endogenous JA (not shown).

4. Discussion

The halo blight symptoms caused by coronatine (1) represent a process of drastically accelerated local senescence which had originally been attributed to the fact

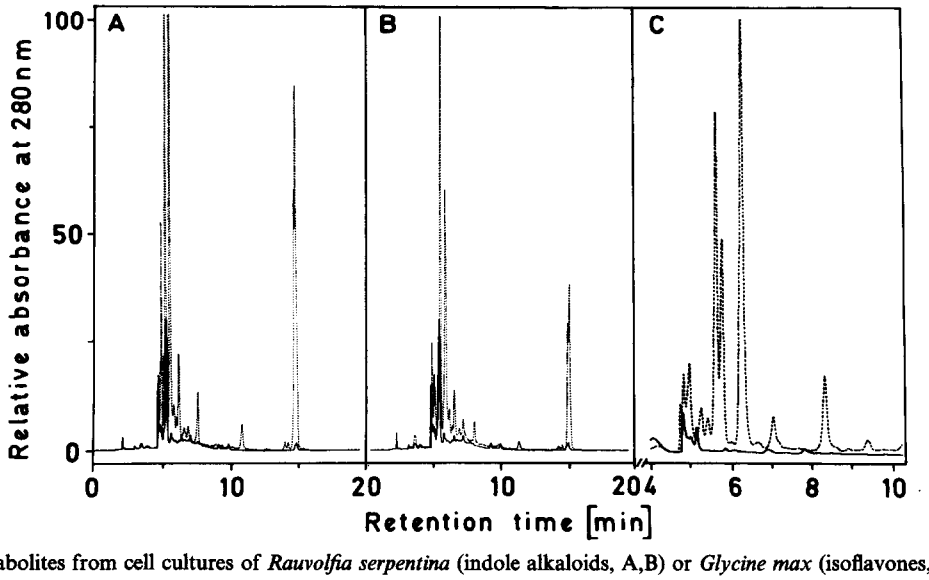

Fig. 4. Secondary metabolites from cell cultures of *Rauvolfia serpentina* (indole alkaloids, A,B) or *Glycine max* (isoflavones, C) treated with 1 μM coronatine (A), 100 μM methyljasmonate (B) or 0.1 μM coronatine (C). Dotted lines: treated tissues, solid lines: control tissues. All experimental conditions as in [13].

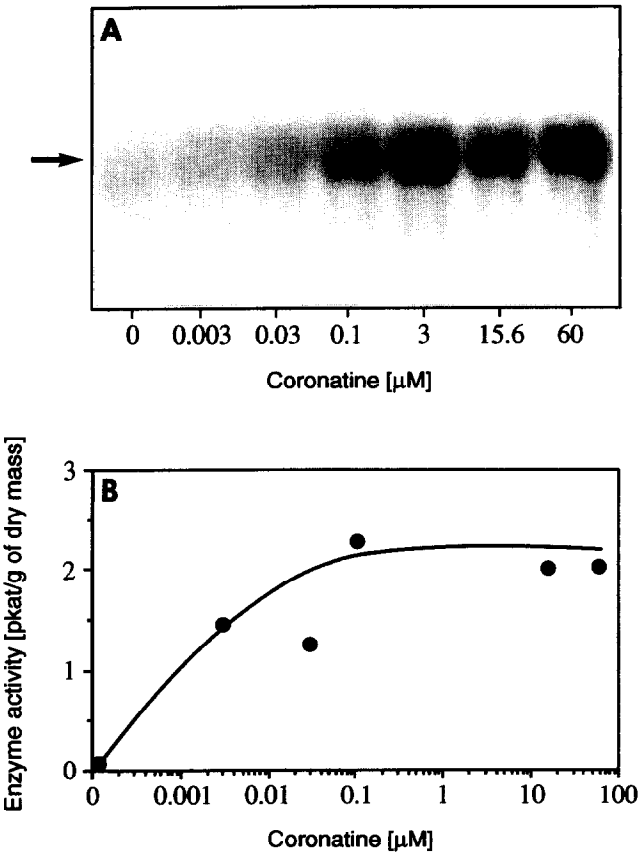

Fig. 5. Induction of berberine bridge enzyme (A) poly(A)$^+$ mRNA and (B) activity in *E. californica* cell suspension cultures in response to treatment with various concentrations of coronatine. To minimize variations in response of the cells, all determinations were made from one culture divided into seven equal portions prior to addition of coronatine (or 100% ethanol in the case of the control) at the indicated concentrations. An aliquot from each culture was allowed to incubate for an additional four days for analysis of benzophenanthridine alkaloids. Total benzophenanthridine alkaloids accumulated were comparable to those values presented in Fig. 3A. Six hours after addition of coronatine, 5 g fresh mass of cells were removed for total RNA isolation and twenty-four hours after inducer addition, 200–250 mg fresh mass of cells were removed for analysis of enzyme activity. Arrow: position of berberine bridge enzyme transcript.

that its non-protein amino acid moiety, coronamic acid, is an ethyl derivative of 1-aminocyclopropyl-1-carboxylic acid (ACC), the direct precursor of the plant senescence promotor, ethylene. It was shown, however, that while it stimulates ethylene production from ACC, coronatine is itself not metabolized to ethylene [4]. The fact that JA, likewise a potent promotor of plant senescence [9], shows some, albeit weak, activity in a coronatine bioassay [7,8], has led to the proposal that the polyketide-derived moiety of coronatine, coronafacic acid (**3**) might be regarded as a JA-analogue [8]. However, coronafacic acid has no activity in coronatine bioassays [6], leaving the toxin's mode of action unresolved.

To investigate this aspect further, we have studied the activity of coronatine in two very different in vivo plant systems specifically responding to jasmonates: (a) the *Bryonia* tendril system where jasmonates substitute for a mechanical stimulus and induce coiling, a complex, dynamic process of differentiation at the cell and organ level [12,14] and (b) the elicitation of secondary metabolite accumulation in plants, a key element of the plant's defense against microbial pathogens, which can be studied conveniently in cell cultures [11,13,19]. In both systems, coronatine was found highly active (Figs. 1–5). The structural requirements for activity of coronatine and a range of its derivatives in the tendril curvature bioassay (Fig. 1) are practically identical to those reported in phytopathological assays [6], suggesting a common structural basis for the two processes. Structural elements essential for activity are (i) the free carboxyl group (the methyl ester of (**1**), (**2**) is nearly inactive, cf. Fig. 1, insert), (ii) the carbonyl function (compound (**5**) being almost inactive), (iii) the presence of the coronafacoyl *as well as* the coronamic acid part in the molecule. Since both coronafacic acid (**3**) and its methyl ester (**4**) were biologically inactive (Fig. 1), coronatine cannot be regarded as a structural analogue of JA/MeJA. In further support of this, the position of the essential carboxyl group of coronatine relative to the cyclopentyl-ring is quite different from that in JA. An overlay of the structures of coronatine and *cis*-12-oxo-phytodienoic acid (**6**), however, reveals a striking similarity of the two compounds (Fig. 6) with the ring systems, side chains, the essential carbonyl and carboxyl functions precisely overlapping in space. Furthermore, C2 to C4 of the octanoic side chain of *cis*-12-oxo-phytodienoic acid, in our model, can be arranged to a bulky, lipophilic structure overlapping with the cyclopropyl-moiety of coronatine. If this is done as in Fig. 6, C5 to C7 of the side chain diverge from the corresponding structural elements in coronatine, i.e. the area of and around the amide bond, to which there is also less similarity in polarity. Otherwise, the arrangement of all charged, polar and lipophilic structural elements of the two molecules match closely. Coronatine must thus be regarded as an analogue of the

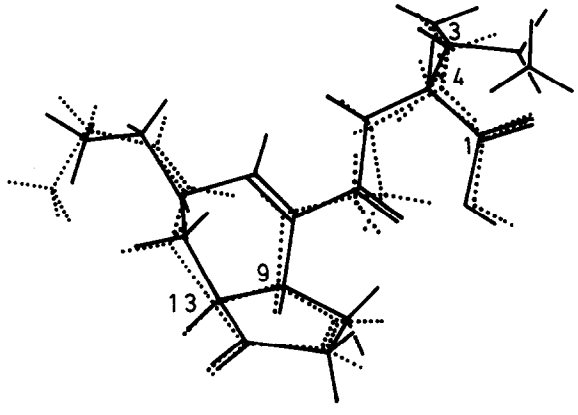

Fig. 6. Molecular overlay of the structures of coronatine (solid lines) and 12-oxo-phytodienoic acid (dotted lines). The numbers refer to 12-oxo-phytodienoic acid and correspond to those in Fig. 2.

octadecanoid precursors of JA. This conclusion is further supported by: (i) the fact that the activity of coronatine and the octadecanoid jasmonate precursors in the tendril system is similar, all three being much more active than MeJA itself (see Fig. 1) and (ii) the fact that the possibility that coronatine induces JA accumulation in the treated tissue could be excluded (e.g. Fig. 3C). Further evidence that coronatine cannot simply be regarded as a JA-analogue came (iii) from the observation that while in some of the biological systems used in our study (Figs. 1 and 4), it was more active than JA, in *E. californica* cell suspension culture, coronatine, even at 100 μM, induced only 40% accumulation of total benzophenanthridine alkaloids, when compared to the same inducing concentrations of JA. At the same time, coronatine was acting at an approximately 50- to 100-fold lower threshold level than was JA (Fig. 3A).

The finding that coronatine is a structural analogue of the pentacyclic octadecanoid precursors of JA without being convertible to it or inducing the synthesis of endogenous JA, yet is active in so diverse JA bioassays, has more general implications for understanding octadecanoid signalling. Our data strongly suggest that cyclic octadecanoids are, by themselves, powerful signal molecules of higher plants and are involved in processes as diverse as mechanotransduction and inducing defense against pathogen attack. The similarity of the jasmonates to animal prostaglandins has been noted [11,20], but our data now suggest much more stunning analogies between lipid-derived signalling molecules of animals and higher plants.

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